# Characterization of Acute 4,4´-Methylene Dianiline Hepatotoxicity in the Rat

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Methylene dianiline (DDM; 4,4´-diaminodiphenylmethane) is a primary aromatic amine that is used extensively in a variety of industrial synthetic processes. DDM is an important intermediate in the production of isocyanates and rigid polyurethane foams and is a component of epoxy hardening agents. Because of its widespread use and large-scale production, there is potential for human exposure and intoxication. A mass intoxication occurred in Epping, England, in the late 1960s. The pathologic changes in exposed individuals were limited primarily to the liver. Liver biopsies revealed portal edema and inflammation with an abundant cellular infiltrate composed of neutrophils, eosinophils, and mononuclear cells (1).

Few investigations of acute DDM-induced hepatotoxicity in animals have been reported. Schoental (2) described the lesions associated with long-term exposure of rats and mice to DDM. These included liver tumors and hepatic lesions consisting of focal midzonal and portal necrosis with marked leukocytic infiltrates. These findings were consistent with the lesions described by Kopelman in humans intoxicated with DDM (1). Pludro et al. (3) carried out a rudimentary evaluation of the acute and subacute toxicity of DDM in rats. Kanz and co-workers (4) characterized the histologic lesions associated with a high dose of DDM. This study revealed early injury to bile duct epithelium, necrosis of hepatic

Methylene dianiline (DDM) is a chemical intermediate in the production of isocyanates and other industrial chemicals, and it is hepatotoxic in humans. The acute hepatotoxicity of orally administered DDM was characterized in rats. Rats receiving DDM (25–225 mg/kg, *per os*) demonstrated a dose-dependent elevation in serum alanine aminotransferase activity, g-glutamyltransferase activity, and serum bilirubin concentration. DDM also caused a decrease in bile flow and an elevation in liver weight. Significant changes in these markers of liver injury occurred between 8 and 12 hr after a single, oral administration of DDM. Histologically, DDM caused multifocal, necrotizing hepatitis with neutrophil infiltration. Changes in the portal regions consisted of bile ductular necrosis, portal edema, neutrophil infiltration, mild fibrin exudation, and segmental necrotizing vasculitis. The role of cytochrome P450 monooxygenase (MO)-mediated metabolism in DDM hepatotoxicity was evaluated using the MO inhibitors, aminobenzotriazole and SKF-525A and the MO inducers phenobarbital and ß-naphthoflavone. Aminobenzotriazole provided protection from DDM-induced hepatotoxicity, whereas SKF-525A had no effect. The effect of phenobarbital pretreatment depended on the dose of DDM administered. At a dose of DDM that produced a maximal hepatotoxic response, phenobarbital did not influence hepatotoxicity. However, phenobarbital pretreatment provided protection against the hepatotoxic effects of a lower dose of DDM. ß-naphthoflavone pretreatment had a more modest effect on DDM-induced hepatic insult. These results demonstrate that DDM causes acute hepatotoxicity in the rat that is dose and time dependent. Results using inducers and inhibitors of MO suggest that DDM requires bioactivation to exert toxicity; however, the relationship between metabolism and toxicity may be complex. **Key words:** aminobenzotriazole, cytochrome P450 monooxygenase, hepatotoxicity, liver, methylene dianiline, ß-naphthoflavone, phenobarbital, rat, SKF-525A. *Environ Health Perspect* 101:130–133(1993)
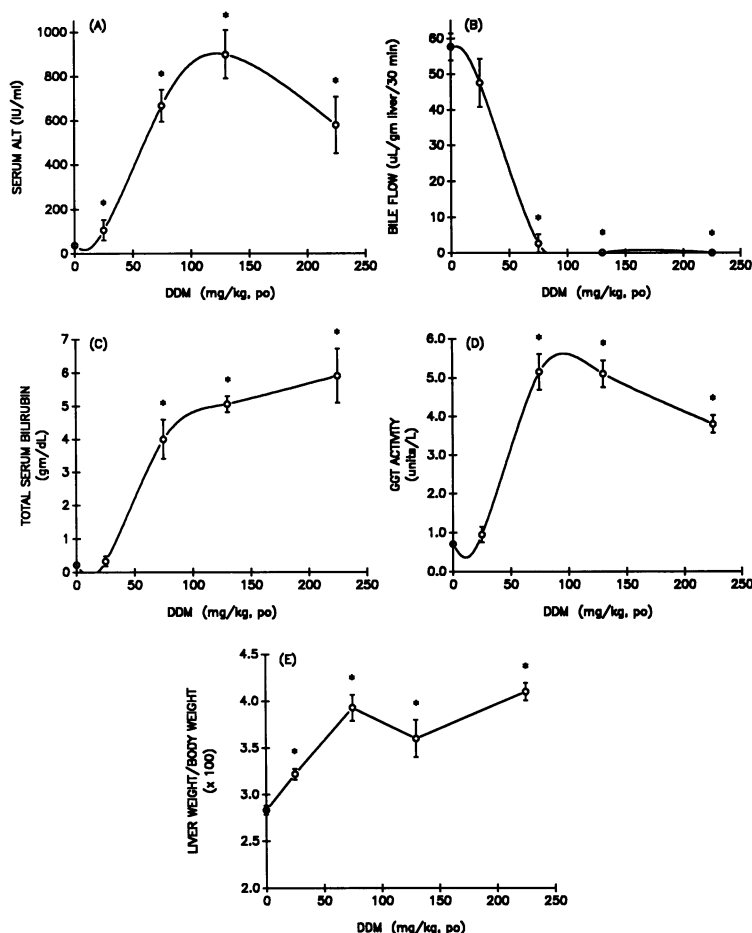
**Figure 1.** Dose-related liver injury after oral administration of methylene dianiline to rats. Results are expressed as the means ± SE. Data were analyzed with a completely random ANOVA or a two-way ANOVA, as appropriate. Comparisons between treatment means were carried out using the Tukey's ω test or the least significant difference test, as appropriate ($6$). The criterion for significance was $p \leq 0.05$. (A) Serum alanine aminotransferase activity; (B) bile flow; (C) total serum bilirubin content; (D) serum γ-glutamyltransferase activity (E) liver weight to body weight ratio ($N$ = 5–6). Error bars, when not present, were too small to appear on the graph. *Significantly different from control.

parenchymal cells, and altered secretion of biliary constituents in rats.

In this study, we further characterized the hepatotoxic effects of DDM. Our aim was to delineate the biochemical and histologic changes associated with acute DDM intoxication and to understand the dose relatedness and development of DDM hepatotoxicity in rats. In addition, we investigated the possibility that the cytochrome P450 monooxygenase system (MO) is involved in DDM-induced liver injury *in vivo*.

Male Sprague Dawley rats [CF: CD (SD)BR; Charles River, Portage, MI] weighing 175–300 g were fasted for 24 hr
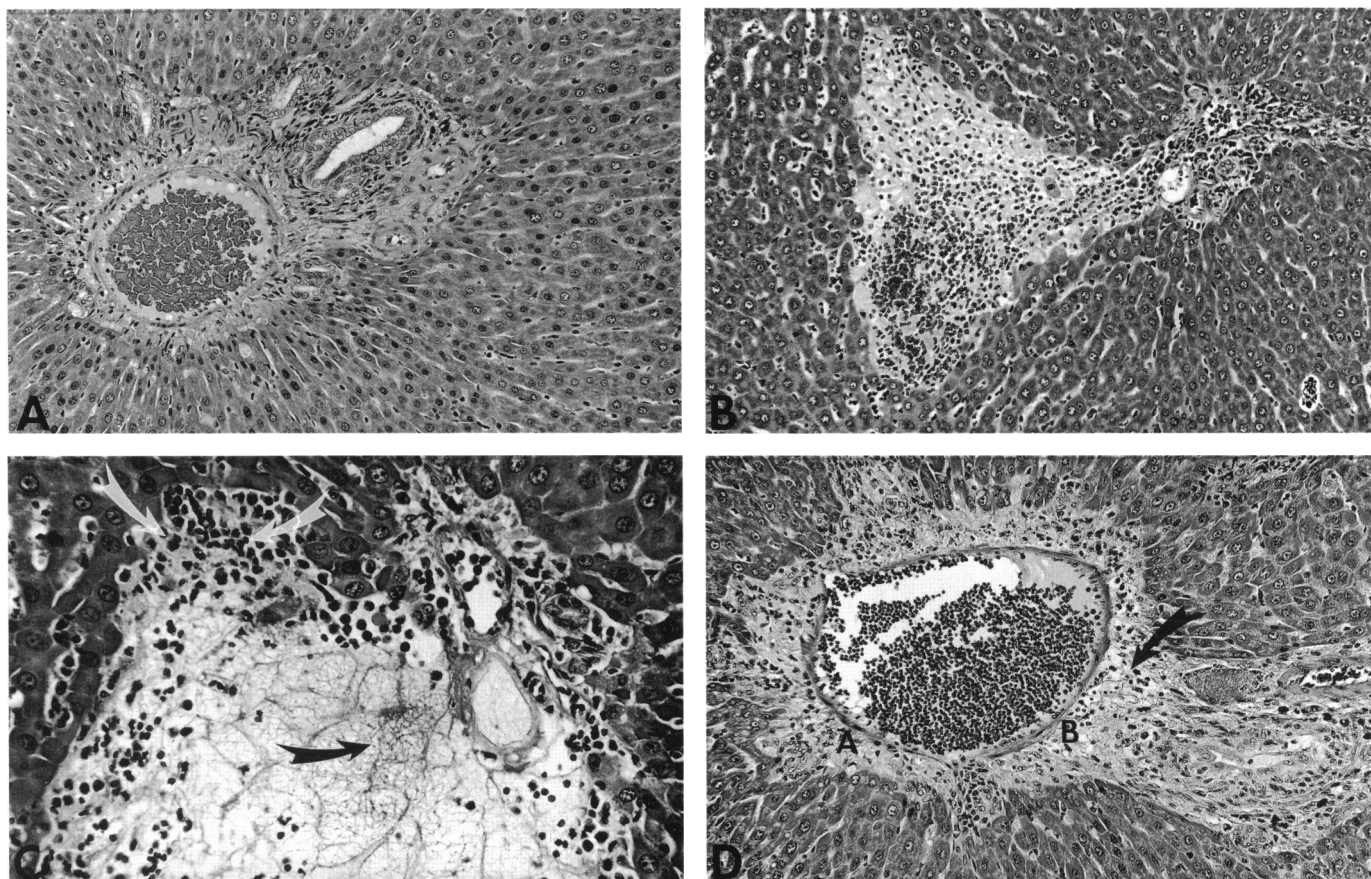
**Figure 2.** Photomicrographs of the livers of rats 24 hr after oral administration of 100 mg/kg methylene dianiline (DDM) or vehicle. (A) Photomicrograph of the liver from a rat receiving corn oil. Note the normal portal tracts and periportal parenchyma. H&E, 129×. (B–D) Photomicrographs of livers from DDM-treated animals. (B) Focus of hepatocellular necrosis extending segmentally from the portal tract and involving the midzonal parenchyma, 128×. (C) Neutrophil infiltration (white arrow) and fibrin exudation (black arrow) within the necrotic hepatic parenchyma, 257×. (D) Periportal edema (arrow), neutrophil infiltration, and segmental necrotizing vasculitis of the portal vein (extending from *A* to *B*), 125×.

before and after receiving DDM. We treated rats with one of several doses (25–225 mg/kg) of DDM (Aldrich Chemical Co., Milwaukee, WI) or an equivalent volume of corn oil *per os*. All doses were administered in a 2 ml/kg volume. We anesthetized the rats with sodium pentobarbital (50 mg/kg, IP) 24 hr after treatment and placed the rats on a heating pad to maintain body temperature. We cannulated the common bile duct as previously described (5) and collected bile flow for 30 min. The rats were then exsanguinated, and blood was collected for determination of indices of liver injury. We quantified total serum bilirubin concentration spec-

trophotometrically (Sigma kit 605-D, Sigma Chemical Co., St. Louis, MO). Gamma-glutamyl transferase (GGT) activity was determined spectrophotometrically by monitoring the GGT-catalyzed formation of *p*-nitroanilide from the substrate, L-γ-glutamyl-*p*-nitroanilide (Sigma kit 419-20). As a marker of hepatic parenchymal injury, we determined serum alanine aminotransferase (ALT) activity via a kinetic spectrophotometric assay (Sigma kit 59-20).

Oral administration of DDM caused a dose-dependent change in all markers of liver injury (Fig. 1). The threshold for toxicity was between 25 and 75 mg/kg DDM.

DDM administration caused concomitant changes in all markers of liver injury measured, including serum ALT, bile flow, serum bilirubin concentration, GGT activity, and liver weight (Fig. 1). Pludro et al. (3) reported that the oral $LD_{50}$ of DDM in rats was 830 mg/kg. They found histologic changes in liver and changes in plasma electrophoretic patterns after subacute administration of DDM at a dose equal to one-tenth of the $LD_{50}$. Our findings are consistent with theirs.

Liver sections from animals that received DDM (100 mg/kg, *per os*) had multifocal lesions consisting of hepatocellular necrosis with hemorrhage and moderate

**Table 1.** Histologic characterization of methylene dianiline (DDM)-induced liver injury

| Time after DDM (hr) | Bile duct necrosis | Edema | Fibrin | Bile ductular neutrophil infiltrate | Portal venous vasculitis | Hepatocellular necrosis | Hemorrhage | Parenchymal neutrophil infiltrate |
|---|---|---|---|---|---|---|---|---|
| 4 | 1.5 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| 6 | 2.5 | 2.0 | 2.0 | 1.3 | 1.3 | 0.5 | 0.0 | 0.0 |
| 12 | 4.0 | 2.0 | 2.0 | 2.0 | 1.5 | 2.3 | 1.3 | 1.5 |
| 16 | 4.0 | 2.0 | 2.0 | 3.0 | 1.8 | 4.0 | 2.3 | 3.5 |

Animals received 100 mg/kg DDM, *per os*. At various times thereafter rats were killed and tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 μM, and stained with hematoxylin and eosin. Histologic changes were assessed from slides that were coded. The pathologist had no knowledge of treatment groups. Liver sections were evaluated for relative severity of lesions present and scored on a 0–4 scale (0 = normal relative to controls, 4 = most severe changes). Control livers demonstrated normal hepatocellular architecture and no pathologic changes. These livers were assigned 0 as an arbitrary score. Three sections of liver from different lobes were evaluated per animal. Each value represents the mean score of 3–5 animals.
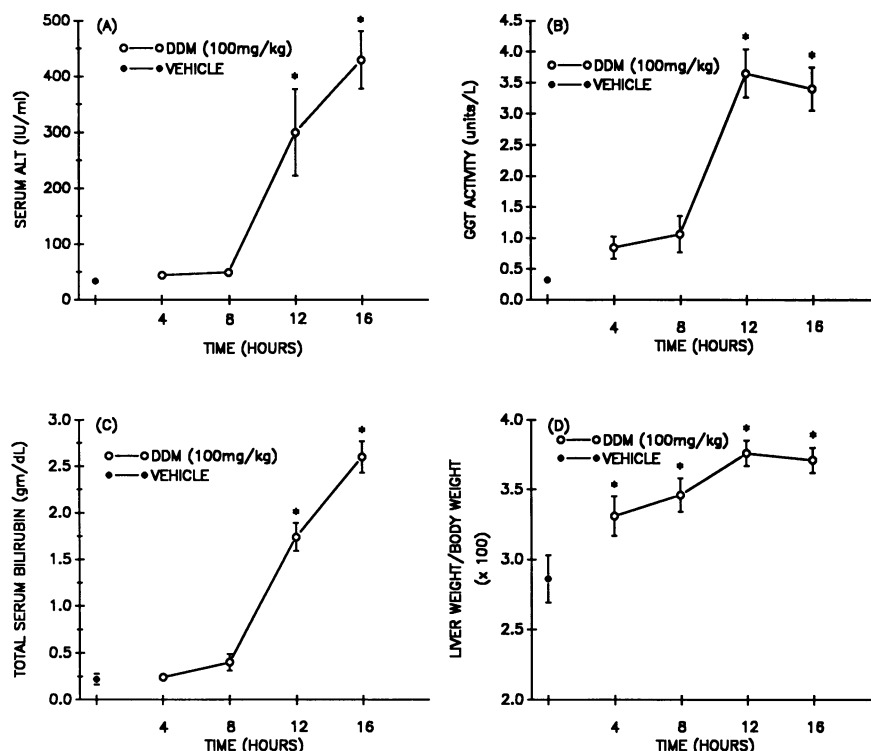
**Figure 3.** Development of acute methylene dianiline (DDM)-induced hepatotoxicity. Fasted rats received 100 mg/kg DDM *per os* or an equivalent volume of corn oil. At 4-hr intervals after administration of DDM, rats were anesthetized with sodium pentobarbital . Blood was collected from the descending aorta, and serum was used for assessment of liver injury by determination of bilirubin concentration, γ-glutamyl-transferase (GGT) activity and alanine aminotransferase (ALT) activity. (A) Serum ALT activity; (B) serum GGT activity; (C) serum bilirubin concentration; (D) liver weight to body weight ratio. Values represent means ± SEM (N = 4–6). Error bars, where not present, were too small to appear on the graph. Zero-time values comprise data from rats killed at various times after vehicle administration. *Significantly different from control value ($p \leq 0.05$).

**Table 2.** Effect of P450 monooxygenase modifiers on methylene dianiline (DDM)-induced hepatotoxicity

| Pretreatment | DDM dose (mg/kg) | ALT (U/l) | Bile flow (µl/g liver/30 min) | Bilirubin (g/dl) | GGT (U/l) | LW/BW |
|---|---|---|---|---|---|---|
| Saline | 100 | 524 ± 65 | 5.5 ± 4.7 | 4.0 ± 0.6 | 5.4 ± 0.6 | 4.1 ± 0.09 |
| ABT | | 145* ± 26 | 26* ± 6.9 | 2.2* ± 0.6 | 2.8* ± 0.4 | 4.2 ± 0.13 |
| | | | | | | |
| Saline | 100 | 540 ± 82 | 0.3 ± 0.1 | 4.2 ± 0.3 | 6.2 ± 0.8 | 3.8 ± 0.07 |
| SKF-525A | | 507 ± 63 | 5.2 ± 3.1 | 4.1 ± 0.3 | 4.6 ± 0.8 | 3.8 ± 0.06 |
| | | | | | | |
| Corn oil | 50 | 279 ± 57 | 27.3 ± 6.1 | 0.6 ± 0.2 | 5.6 ± 0.9 | 3.7 ± 0.10 |
| BNF | | 136* ± 31 | 30.9 ± 3.4 | 0.4 ± 0.1 | 2.8* ± 0.3 | 4.5* ± 0.08 |
| | | | | | | |
| Saline | 50 | 588 ± 122 | ND | 3.0 ± 0.7 | 6.6 ± 1.0 | 3.6 ± 0.18 |
| Phenobarbital | | 88* ± 11 | ND | 0.1* ± 0.01 | 0.8* ± 0.2 | 3.9 ± 0.07 |
| | | | | | | |
| Saline | 100 | 823 ± 100 | 3.9 ± 1.5 | 4.6 ± 0.5 | 10.0 ± 1.8 | 3.9 ± 0.05 |
| Phenobarbital | | 706 ± 138 | 7.0 ± 4.5 | 3.2 ± 0.7 | 8.2 ± 1.6 | 4.7* ± 0.1 |

Abbreviations: ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; LW/BW, liver weight to body weight ratio; ABT, aminobenzotriazole; BNF, ß-napthoflavone; ND, not detected.
Two hours before administration of DDM (100 mg/kg in corn oil, *per os*), rats were treated IP with 40 mg/kg SKF-525A (20 ml/kg in saline), Sigma Chemical Co., St. Louis, MO), 100 mg/kg ABT (2 ml/kg in saline), or an equal volume of saline vehicle. Twenty-four hours later rats were anesthetized, bile flow was assessed, serum was obtained for evaluation of biochemical markers of liver injury, and liver sections for histologic evaluation were taken as described above. Phenobarbital (70 mg/kg in saline, IP), or ß-naphthoflavone (80 mg/kg, IP as a suspension in corn oil, Sigma), or their respective vehicles (2 ml/kg) were administered daily for 3 days preceding administration of DDM (100 or 50 mg/kg, *per os*). Twenty-four hours after DDM administration, rats were processed as described above. Values represent means ± SEM, n = 8–11. *Significantly different from pretreatment controls ($p \leq 0.05$).

neutrophil infiltration (Fig. 2). The necrosis involved segments of periportal hepatocytes but did not surround portal tracts. Frequently, the parenchymal insult extended into the midzonal regions of hepatic lobules. The lesions associated with the portal triad consisted of bile ductular necrosis, portal edema with fibrin exudate, and neutrophil infiltration. A segmental necrotizing vasculitis of the portal vein was also evident. The earliest change identified with the hepatotoxicity of DDM was bile ductular necrosis, and histologic markers of liver injury continued to increase in severity over the course of 16 hr (Table 1). Histologic analysis of livers from animals receiving corn oil vehicle demonstrated normal hepatic histology with no apparent lesions.

Several investigators have described the distribution of histologic changes in liver associated with DDM intoxication (*1,2,4*). In rats and mice, the loss of parenchyma appeared to be primarily midzonal (*1,2*) or midzonal and periportal (*4*). Our observations were generally similar to those reported by these investigators. However, we found that the parenchymal lesions appeared adjacent to portal regions and extended into midzonal regions of the liver. The lesions did not appear to be strictly periportal in that they did not encircle the portal areas. In addition, the lesions were not restricted to the midzonal areas of the liver lobule, and they varied in distribution. Finally, we observed a prominent necrotizing vasculitis, which has not been reported previously. This event occurs early and therefore might be important to the mechanism of action of DDM.

Recently, Kanz and co-workers (*4*) evaluated some of the early hepatic changes associated with administration of a large dose (250 mg/kg) of DDM. An early (30 min) increase in biliary protein and decreases in bile salts and bilirubin content in bile occurred. Elevations in bile flow and bile glutathione content were concurrent with these changes. Changes in serum markers of hepatic parenchymal injury and cholestasis were apparent by 4–8 hr. Histologic evidence from the present study suggests that the earliest lesion is probably biliary in nature (Table 1), a finding consistent with the results of Kanz and co-workers (*4*). The onset of liver damage caused by DDM appears to be somewhat delayed in expression. Although subtle changes in the histologic appearance of the portal regions in the liver are evident by 4–8 hr, the first significant changes in biochemical markers of liver injury do not occur until later (Fig. 3).

The lesions of DDM intoxication have common histologic features to those

caused by another cholangiolitic hepato-toxicant, α-naphthylisothiocyanate (ANIT). Portal edema, biliary epithelial injury, and inflammation occur in both models. However, the histologic distribu-tion of lesions caused by ANIT are almost exclusively limited to periportal areas. Moreover, a distinct hepatic vascular injury has not been reported after ANIT adminis-tration. DDM seems to cause a more severe hepatic parenchymal lesion and gen-erates a more marked inflammatory re-sponse than ANIT.

In a preliminary report, Ansari and co-workers (7) presented evidence suggesting the presence of metabolites of DDM in rat urine. Kajbaf and co-workers (8) have recently identified oxidized metabolites of DDM from microsomal preparations of rabbit liver. To gain insight to the role of metabolism in DDM hepatotoxicity, ani-mals were pretreated with various agents at doses that have been shown to alter MO activity and to influence hepatotoxicity of agents that require bioactivation by this enzyme system (9–12). Aminobenzotria-zole (ABT), an inhibitor of MO, was effec-tive at ameliorating the hepatotoxic effects of DDM, as demonstrated by attenuation of all markers of injury evaluated (Table 2). However, the MO inhibitor SKF-525A did not protect against DDM-induced liver damage. The difference in the results obtained when using ABT ver-sus SKF-525A may reflect a difference in the spectrum of cytochrome P450 iso-zymes inhibited by the two agents (10, 12,13). It is plausible that SKF-525A did not inhibit the specific isozyme that bioac-tivates DDM, whereas ABT effectively attenuated metabolic activation.

The effect of phenobarbital pretreat-ment varied with the dose of DDM. At a dose of DDM (100 mg/kg) that caused maximal hepatotoxic effects (Fig. 1), pre-treatment with phenobarbital had no effect on any of the markers of liver injury (Table 2). At a more moderate dose of DDM (50 mg/kg), phenobarbital pretreatment atten-uated the hepatotoxicity. This result is generally consistent with the preliminary results of Kanz et al. (14), who found that toxic effects of a large dose of DDM were only modestly influenced by phenobarbital pretreatment, and protection was largely restricted to extrahepatic effects of DDM. We also evaluated the effect of pretreat-ment with a second inducer of MO activi-ty, ß-napthoflavone (BNF). This agent had a modest effect on DDM hepatotoxic-ity (Table 2). Like phenobarbital, BNF pretreatment did not affect the toxicity of a higher dose (100 mg/kg) of DDM. These findings suggest that there is a cytochrome P450-dependent detoxification pathway for DDM. However, it should be noted

that phenobarbital has myriad effects, not only on phase 1 MO metabolism but also on other enzyme systems. Thus, the atten-uation of DDM toxicity may reflect an action on phase 1 metabolism of DDM or on other metabolic systems, such as phase 2 conjugating systems, which might con-tribute to DDM hepatotoxicity.

Assuming that the various agents we used act by influencing metabolism of DDM by MO, the results are consistent with the following hypothesis: DDM requires metabolic activation for its toxici-ty. This activation is carried out by an isozyme of cytochrome P450 that is inhib-ited by ABT but not by SKF-525A. In addition, there is a competing, P450-medi-ated reaction that is important in the detoxification of DDM. This competing reaction is enhanced by phenobarbital pre-treatment and to a lesser extent by BNF pretreatment. At low DDM doses, the two opposing pathways compete for DDM substrate. Accordingly, an increase in the detoxification pathway by phenobarbital or BNF pretreatment leads to decreased for-mation of toxic metabolites by the compet-ing bioactivation pathway. At higher DDM doses, enough substrate is present to saturate the bioactivation pathway irrespec-tive of the action of the detoxification pathway. There is precedent for the con-cept of competing, toxifying, and detoxify-ing pathways for hepatotoxicants in the cases of acetaminophen and bromobenzene (13,15). Clearly, further study, including the identification of DDM metabolites in the rat, is needed to test this hypothesis completely.

In summary, our results indicate that DDM causes dose- and time-related toxici-ty classified as multifocal, cholangiolitic hepatitis, the lesions of which are distrib-uted in portal and midzonal regions of liver lobules. The results with inducers and inhibitors of cytochrome P450 mono-oxygenase suggest that DDM requires metabolic activation for its toxicity. As a histologic feature of DDM hepatotoxicity is a marked inflammatory response, it would be of interest to evaluate the role of inflammatory cells and soluble mediators in DDM-induced liver injury.

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